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The Surface Events at Fertilization: The Movements of the Spermatozoon Through the Sea Urchin Egg Surface and the Roles of the Surface Layers

Gerald Schatten and Daniel Mazia

Department of Zoology, University of California, Berkeley, CA 94720

The sea urchin egg surface at fertilization has been examined with the scanning electron microscope to reveal the movements of the spermatozoon from the exterior, through the surface layers, and into the egg cytoplasm. The layers that the spermatozoon encounter have been studied to determine their physical and chemical natures and their role in early development.

By studying the outside of whole eggs and the inner face of surfaces isolated shortly after fertilization, it has been possible to compile data on the movements of the spermatozoon through the egg surface. The spermatozoon initially contacts the egg with the elongated acrosomal process. The vitelline sheet, the outermost layer of the egg, separates slightly next to the attached spermatozoon. As membrane fusion between the gametes occurs, the plasma membrane from the egg engulfs the spermhead, the cortical granules start to discharge their contents, and a spreading surface deformation, concommitant with a distortion of the fibrous cortex, is initiated. A cluster of elongate microvilli surround the perpendicularly fusing spermatozoon. These microvilli interdigitate as the spermatozoon is forced to lie upon the egg surface between the plasma membrane and the matrix of cortical fibers. The spermatozoon then rotates additionally to enter the egg cytoplasm with the posterior end first; it has rotated 180° through the cell surface. Finally, it detaches into the egg cytoplasm, leaving a scar in the cortex through which it penetrated.

The egg cortex, previously unobserved by electron microscopy, is revealed to be composed of 50–200 nm fibers. At fertilization they are uniformly organized but during later development this order is lost. The cortex is from 0.2–0.5 μm thick and is a contractile structure.

The role of the outer surface in releasing the cell from the metabolic constraints of the unfertilized egg is shown, and the apparent differences in the mobilities of the membranes derived from the sperm and from the egg are demonstrated. The relation of these layers to the movements of the spermatozoon, to the activation of the egg, to the block to polyspermy, and to each other are discussed.

Key words: fertilization, cell surface, membrane fusion, cortex, cell fusion

". . . . the initiation of development in the sea urchin egg is due to a change at the surface of the egg – apparently a cytolysis of the cortical layer which results generally in membrane formation."

– Jacques Loeb, 1913 (1).

INTRODUCTION

Fertilization of the sea urchin egg is a unique system for the study of cell surface phenomena. At fertilization, after the fusion of the acrosomal vesicle with the plasma membrane of the spermatozoon, the plasma membranes of the 2 gametes fuse, initiating a

series of complex surface reactions. The permeability of the plasma membrane undergoes a series of changes, as evidenced by electrophysiological data (2). Additionally, the membrane changes from a fusible state to a nonfusible state, to prevent the entry of supernumerary spermatozoa. The cortical granules which lie beneath the plasma membrane fuse with the membrane and discharge their contents. This secretion, containing structural and enzymatic components (3), dramatically alters the outer surface structure of the egg, i.e., all the structure peripheral to the lipid bilayer.

Prior to insemination the egg was invested with a vitelline sheet which accurately followed the contour of the short array of microvilli. With the secretion of the cortical granules, the vitelline sheet is elevated and laminated with their contents to form the thicker fertilization coat. Additionally, certain peripheral membrane components, which attach the vitelline sheet to the plasma membrane, are detached, resulting in the metabolic turn-on of the egg (4). Simultaneous with these events, a surface deformation, probably caused by a contraction of the cortex, spreads over the egg surface. The membranes derived from the gametes intermix as the sperm nucleus, midpiece, and axoneme penetrate through the cortex to the egg cytoplasm. Eventually, the male and female pronuclei fuse to form the zygote nucleus required for proper development (Table I).

This unusual series of radical surface alterations can serve as a model for examining surface changes found in most other cells. The phenomena of membrane fusion, cell fusion, and secretion, and the properties, roles, and interactions of the cortex and glycocalyx with respect to the plasma membrane can be analyzed in relation to the life-history of the cell. In addition to the successful dissection of outer surface components (by both physical and chemical means), the entire surface, i.e., the vitelline sheet, the membrane, the cortex, has been isolated, and morphological examinations of both the outer surface (in intact eggs) and of the inner surface with these isolated preparations is now possible.

The chain of surface events occurring at fertilization is examined in this report with the scanning electron microscope (SEM), beginning with the attachment of the elongated acrosomal process, through membrane fusion between the gametes, the subsequent penetration and rotation of the spermatozoon, and the detachment of the membraneless spermatozoon from the egg cortex into the cytoplasm. The response of the surface and the cortical reactions accompanying this penetration are described, as well as the appearance of the cortex after completion of the cortical granule discharge. It has been possible to infer information about the relative fluidity of the membranes using naturally-occurring cell-surface markers. The properties of the layers that the spermatozoon must penetrate on its journey to the cytoplasm are described, and speculations regarding their role in early development and at fertilization are discussed.

THE MOVEMENTS OF THE SPERMATOZOON

In this section information about the entry, penetration, rotation, and detachment of the spermatozoon from the California sea urchin Strongylocentrotus purpuratus has been compiled from examinations of the outer surface of whole eggs and of the inner side of surfaces isolated shortly after insemination (see [5] for details). The external images were obtained by gluing eggs to a polylysine-coated plate (6), adding sperm, and rapidly transferring the plate into a glutaraldehyde fixing solution. The gluing step reduced the area on the egg surface available for sperm penetration. Only the tops and side of the egg are accessible to the spermatozoon; this is precisely the region viewable in the scanning electron microscope (SEM). Furthermore, this gluing process permits very rapid fixation,

TABLE I. Summary of the Surface Layers of the Egg and Their Properties at Fertilization

Layer	Alteration at Fertilization	Function at Fertilization
The vitelline sheet	Detaches from the egg surface at fertilization and elevates to form the fertilization coat.	Sperm binding (?); block to polyspermy (?); holds the unfertilized microvilli in a uniform array.
The metabolic derepressor (= the connection between the vitelline sheet and the plasma membrane)	Detaches from the egg surface at fertilization and during metabolic turn-on.	Metabolic derepressor of the egg, its release frees the unfertilized egg from its metabolic constraints; involved in permanent block to polyspermy.
The plasma membrane	Changes in resistances to ions; the sperm plasma membrane and cortical granule membranes are added at fertilization.	Altered ionic permeabilities result in changed membrane potential; after fusion with a spermatozoon, it resists further attempts to prevent entry of supernumerary sperm; permits discharge of the cortical granule contents and the entry of the sperm nucleus, midpiece, and axoneme.
Microvilli	Elongation and disarrayal after fertilization and metabolic turn-on.	Increased surface area for transport (?); shape correlated with metabolic activity.
The cortex	Contracts as the spermatozoon penetrates the egg surface.	This contraction results in the deformation of the outer surface; it probably clusters the microvilli around the entering spermatozoon, and may aid in the discharge of the cortical granules and in the rapid block to polyspermy.
The cortical granules	At fertilization they fuse with the egg plasma membrane, discharging their contents into the perivitelline space.	The structural contents of the cortical granules laminate the undersurface of the vitelline sheet and elevate and harden it to form the fertilization coat. The enzymes released detach unsuccessful sperm, and may aid in metabolic activation and the block to polyspermy. The addition of the cortical granule membranes to the egg plasma membrane may alter the properties of this membrane.

since only a minimal volume of suspending fluid is brought over into the fixative. Finally, multiple plates could be simultaneously inseminated and fixed at accurate time points to capture the crucial events.

The observation of the inner face of the unfertilized egg surface was first performed by Vacquier (7), who was able to shear off the tops of attached eggs in calcium-free sea water. The remaining bottom surface, when examined in the SEM, revealed the cortical granules associated with the inner surface of the egg. This method for observing the inner surface is not suitable for examining the entry of the spermatozoon, since the upper half of the egg is sacrificed during the isolation. Furthermore, after fertilization the surface of the cell is separated from the fertilization coat; shearing then will detach the egg.

For these reasons, the observations of the events of fertilization as seen on the inner aspect of the cell surface were made on cell surface complexes isolated immediately after insemination. Here the eggs are inseminated, quickly transferred to the isolation medium (0.3 M KCl, 0.35 M glycine, 2 mM EGTA, 2 mM $MgCl_2$, pH 7.5), and gently cracked to open up the surface; the entire egg surface is maintained. The surface complex, from which the internal contents of the cell are washed away, is glued to a polylysine-coated plate and fixed. Since it always attaches by the outer surface, it is possible to observe the irruption of the spermatozoon through the inner surface. A series of scanning electron micrographs depicts the inner surface of the egg from the moment of membrane fusion, as the sperm travels through the surface, to its appearance on the cortex, and finally the detachment of the spermatozoon from the cortex into the egg cytoplasm.

The External Events

The first image (Fig. 1) depicts an egg attached to the polylysine-coated plate while the sperm are in the process of attempting fertilization. The sperm can only bind to the tops and sides of the egg since the bottom is affixed to the polycationic substrate. The earliest event of fertilization, the discharge of the acrosome of the spermatozoon, is seen in Fig. 2. The resulting filament has a tip thickness of about 100 nm and a basal diameter of 300 nm; it extends about 3 μm in length. A vesicle, probably derived from the sloughed-off membrane at the time of fusion of the acrosomal vesicle with the plasma membrane (8), can be observed attached to the base of the spermhead. The acrosomal process appears to shorten and thicken as the spermatozoon approaches the egg surface. Figure 3 reveals the perpendicular attachment of the spermatozoa to the vitelline short of the egg. The unfertilized egg is characterized by the regular array of short microvilli; these microvilli elongate and become disarrayed after fertilization or metabolic turn-on.

Prior to the moment when clearly fused areas between the sperm and egg membranes can be observed, the vitelline sheet nearest to the tip of the spermhead appears

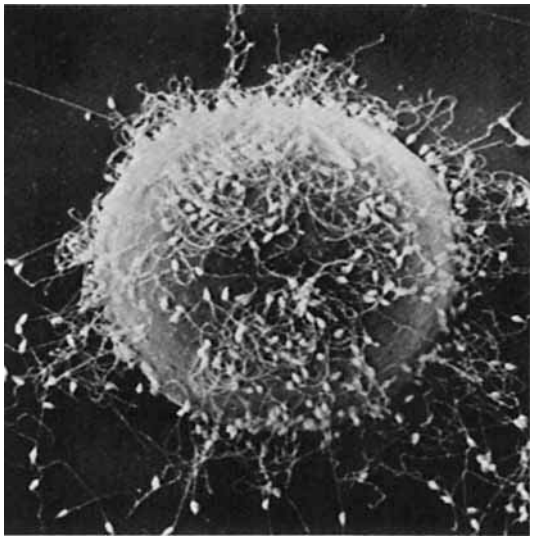

Fig. 1. An early stage of insemination of an egg glued to a polylysine-coated slide. Only the tops and sides of the egg are available for sperm binding. 1,000 X.

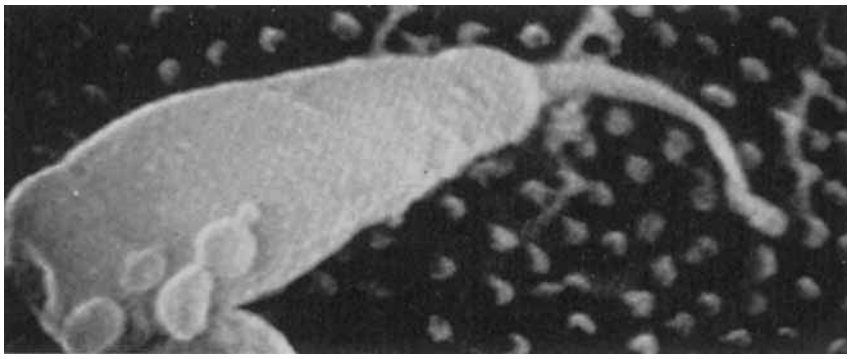

Fig. 2. The sperm initially contacts the egg by the elongated acrosomal process. These processes appear thicker and shorter as the spermhead approaches the egg surface. 20,000 ×.

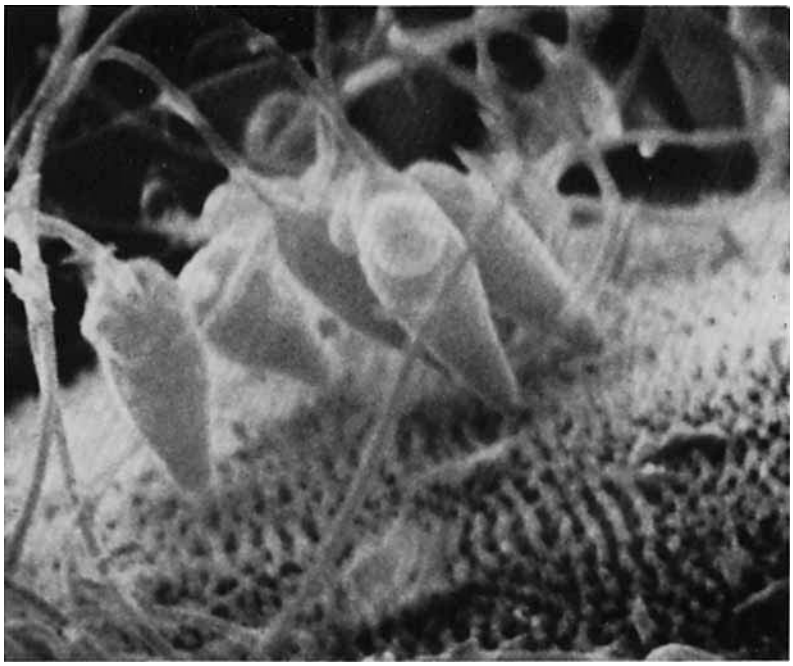

Fig. 3. The sperm attach perpendicularly to the vitelline sheet of the egg surface. The short, arrayed microvilli are characteristic of an unfertilized egg. 10,000 ×.

torn (Fig. 4). At this stage, 50 nm strands, probably originating from the vitelline sheet, are associated with the spermatozoon. These strands will persist until the spermatozoon disappears from the outer surface. Notice the slight bulge in the egg surface at the site of contact with the spermatozoon; this will become quite pronounced. Then, as fusion occurs, the egg surface at the fusion site bulges up about 1.5 μm (Fig. 5). The spermatozoa are almost always held in a perpendicular fashion during membrane fusion as well as during the initial attachment stages. It is important to observe that the membrane from the spermatozoon can easily be distinguished from that of the egg. The sperm membrane is smooth, with no microvilli, whereas the egg is covered with microvilli. As fusion progresses,

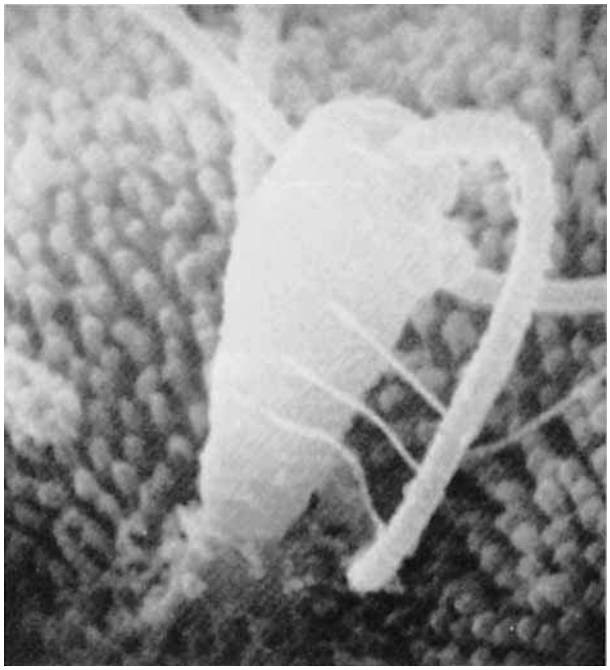

Fig. 4. The vitelline sheet adjacent to the anterior tip of the spermhead appears torn or solubilized just prior to the moment of membrane fusion. Fifty nm strands connect the egg surface. 20,000 ×.

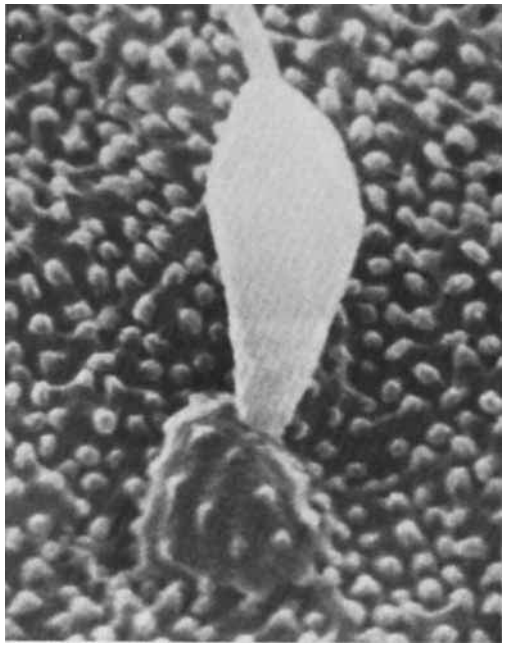

Fig. 5. The egg surface bulges at the earliest stage of membrane fusion. The egg surface can be distinguished from that of the spermatozoon by the presence of microvilli. 15,000 ×.

the membrane surrounding the anterior part of the sperm has microvilli, an indication that this membrane is derived from the egg (Fig. 6). The loose appearance of the smooth membrane covering the distal regions of the sperm is consistent with appearance of egg membrane covering the anterior areas. The 50 nm strands can again be observed with the spermatozoon. Additionally, there is a slight surface deformation. This deformation will spread in diameter and magnitude as penetration occurs.

As the spermatozoon begins its entry, the loosely covering plasma membrane develops a convoluted appearance. The egg plasma membrane appears further up on the spermhead, again using the microvilli as an egg membrane marker. The microvilli on the egg surface cluster around the spermatozoon and elongate from the stubby unfertilized state (150 nm diameter, 300 nm long) to form processes of the same diameter up to 1 μm long. The basal diameter of the cluster of microvilli is 2.5 μm and is composed of about 20 elongate microvilli (Fig. 7). In Fig. 8 the elongate microvilli can be observed to cluster around the spermatozoon as it begins to penetrate into the egg surface. Then these microvilli interdigitate over the spermhead as it enters further into the egg (Fig. 9). It is interesting that these elongate microvilli are bent, and curl over the entering spermhead.

The microvilli are resorbed as the sperm penetrates further into the egg. In Fig. 10 it will also be observed that the membrane slackness previously associated with the region around the spermhead is now resorbed.

As the spermatozoon further penetrates into the egg, the papillae of the vitelline sheet closest to the spermhead develop indentations in their tips, and the 50 nm strands radiate from the penetration site (Fig. 11). The egg surface has been becoming more deformed, and this deformation now covers several μm. The bulging of the egg membrane is now completely resorbed. Figure 12 is a side view demonstrating that the spermatozoon has already undergone a rotation. Notice that the spermhead and midpiece are lying on the egg surface. Finally the spermhead and midpiece are pulled out of view, leaving the tail protruding as the fertilization coat elevates (Fig. 13).

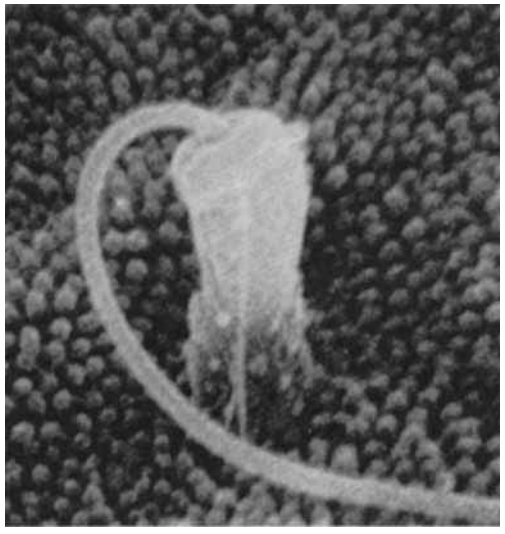

Fig. 6. A later stage of membrane fusion. The membrane derived from the egg now surrounds the anterior portion of the spermhead. 10,000 ×.

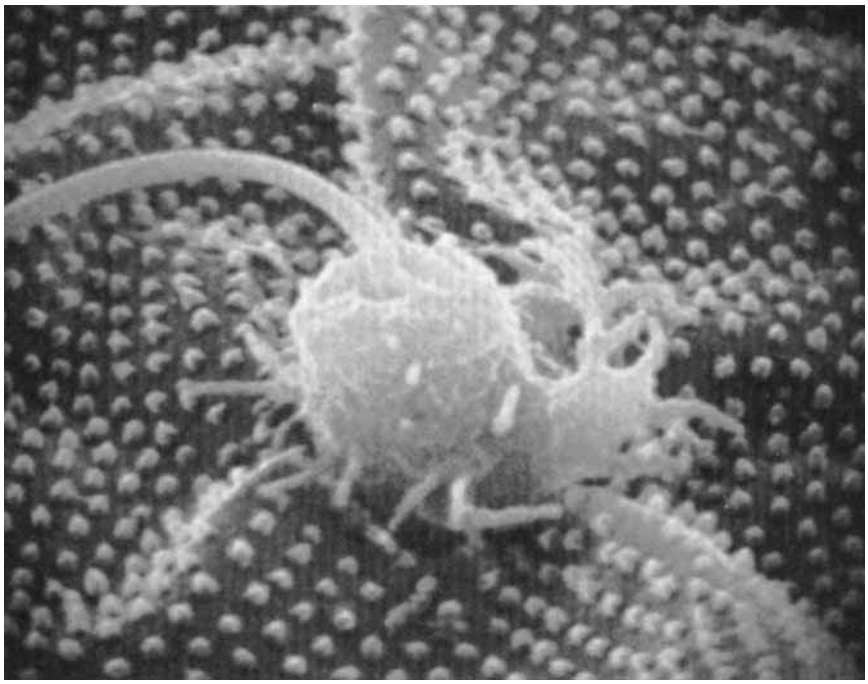

Fig. 7. The egg membrane continues to rise around the spermhead. Microvilli elongate around the spermatozoon as the membrane derived from the sperm appears slack and convoluted. 15,000 ×.

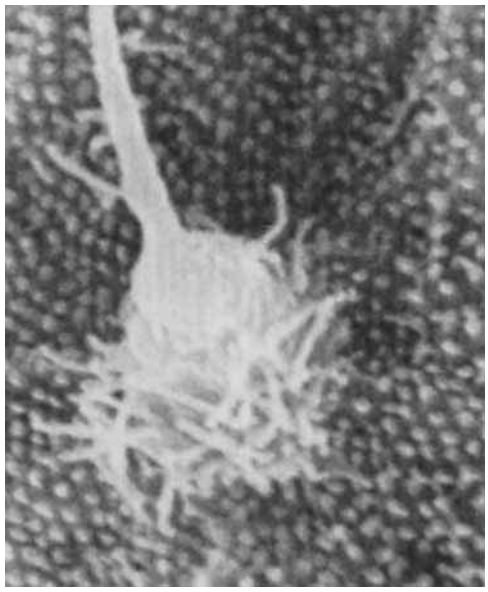

Fig. 8. The elongate microvilli cluster around the spermhead as the spermatozoon begins its entry. 10,000 ×.

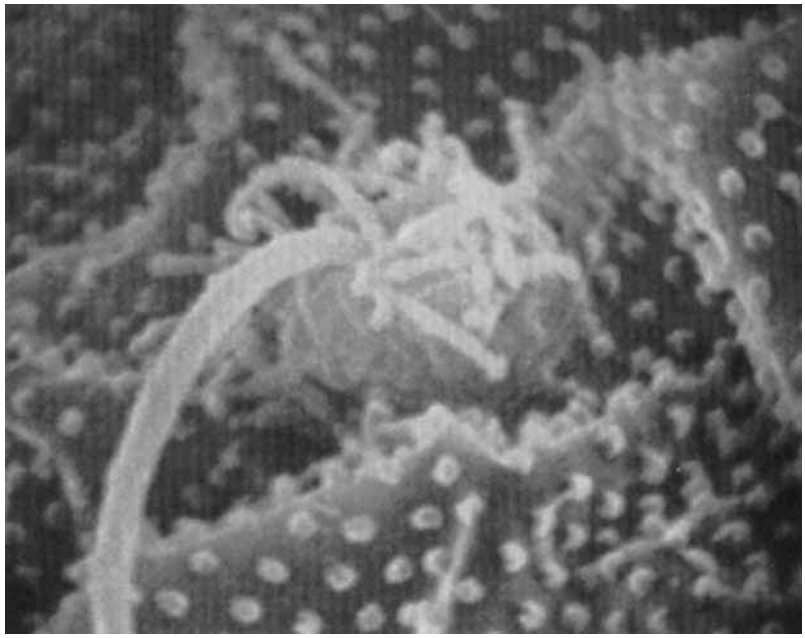

Fig. 9. These elongate microvilli interdigitate over the spermatozoon. 20,000 ×.

Fig. 10. The elongate microvilli are resorbed as the slackness of the membrane around the spermatozoon is taken up. 10,000 ×.

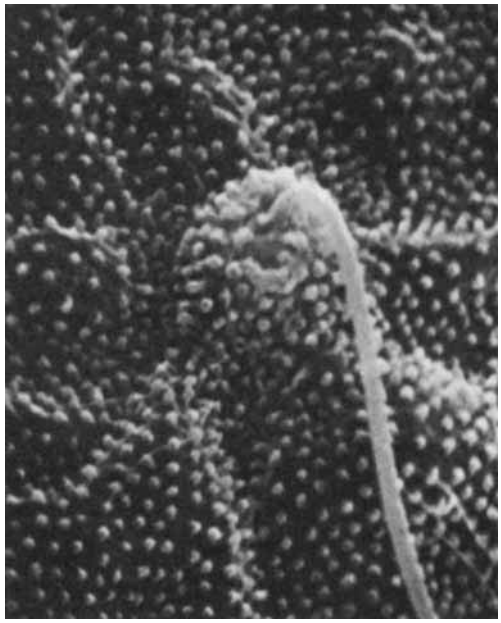

Fig. 11. The head of the spermatozoon has almost completed its entry as the deformation on the surface in the region of the sperm has spread. The indentations in the tips of the papillae of the vitelline sheet around the entering spermatozoon may have resulted when the underlying microvilli were withdrawn. The 50 nm strands now radiate from the penetration site. 11,250 ×.

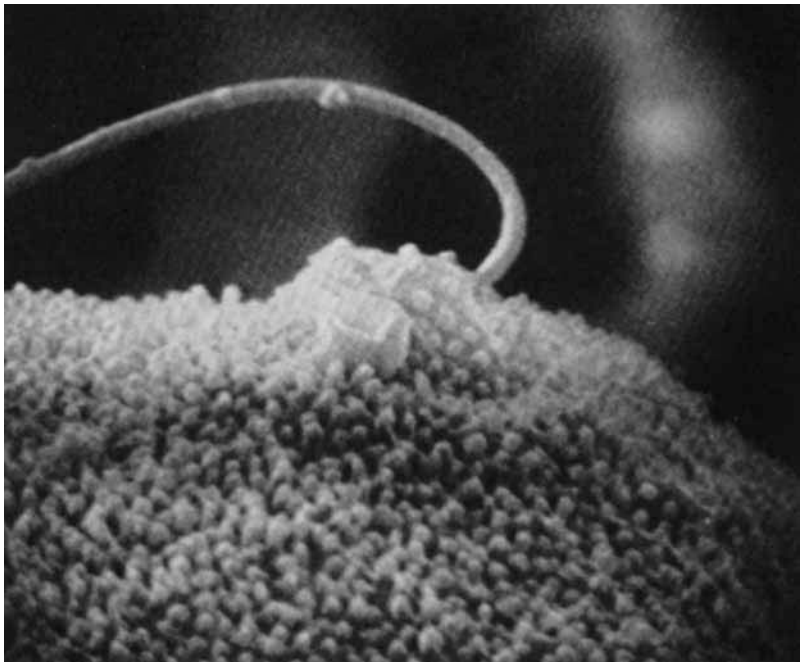

Fig. 12. This side view demonstrates that the spermatozoon has rotated so as to lie horizontally to the egg surface. 15,000 ×.

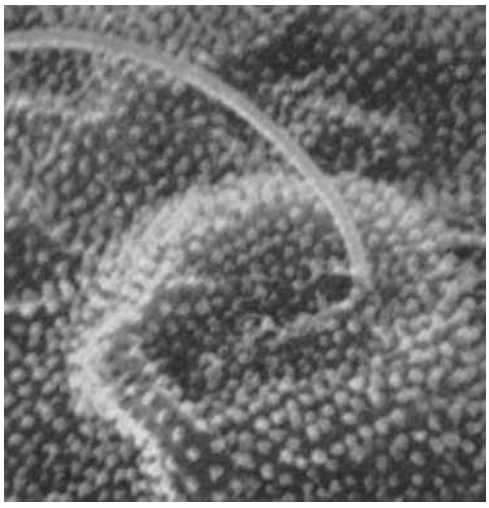

Fig. 13. Finally, the spermhead and midpiece are pulled from view, leaving only the spermtail protruding as the fertilization coat starts to elevate. 10,000 ×.

The Inner Surface Events

The phase contrast micrograph of the isolated egg surface (Fig. 14) demonstrates that the empty surface tends to maintain the spherical shape of the egg. This visual evidence of the rigidity of the surface structure fits well with biophysical evidence, e.g., deformability measurements on egg (9). The underlying blanket of cortical granules can also be observed as dark spheres. Since only the outer surface of such preparations will glue to the polylysine-coated plate, the inner surface faces up and is rendered viewable in the SEM.

The low magnification scanning electron micrograph (Fig. 15) reveals the inner and outer aspects of a glued egg surface, as well as the local discharge of the cortical granules surrounding the site of the entry of the spermatozoon. Since the sperm initiates a spreading wave of cortical granule discharge at fertilization, it is possible to determine the relative time after fertilization of the isolated surface. The larger the patch devoid of cortical granules, the more time since cortical granules discharge occurred, and the longer the time between membrane fusion and isolation of the surface. By measuring the size of these patches it is possible to sequence the events on the inside surface, beginning from the moment of membrane fusion, through the appearance of the spermatozoon, its subsequent rotation, and, finally, its detachment from the egg surface into the cytoplasm.

The first change on the inner surface associated with fertilization is the appearance of a patch devoid of the 1.13 μm cortical granules (Fig. 16). In the center of this patch small particles (235 nm) are clustered in a circle with a diameter of 2.6 μm. The cortex is observed here as covering the inner surface with 50–200 nm fibers which circumscribe hexagonal polygons. A thickening of the cortex radiates from these clustered particles; this distortion extends in 3 roughly equilateral directions. The small particles cluster in Fig. 17, and the thickening in the cortical network is greater and covers a larger area. Triangular networks can still be seen.

Next, a bulging near the center of the patch occurs (Fig. 18). This is probably the spermatozoon "caught" in the egg surface. Figure 19 is an unambiguous image of the membraneless spermatozoon lying between the cortex and the plasma membrane;

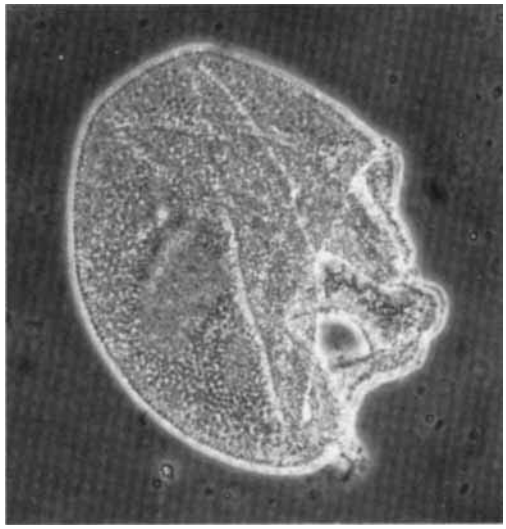

Fig. 14. A phase contrast micrograph of an isolated surface complex. Although empty, these surfaces tend to maintain the spherical shape of the egg. The cortical granules can be observed as the small granules underlying the egg surface. 1,000 ×.

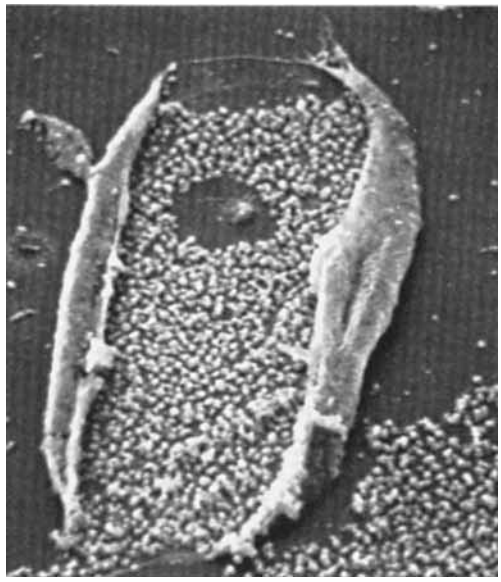

Fig. 15. Low power SEM view of a surface isolated shortly after fertilization. The cortical granules are discharged around the site of sperm entry. The outer surface can be observed at the right and left where the surface is folded over. 1,000 ×.

it is still separated from the egg cytoplasm by the fibrous cortex. Notice that the membraneless spermatozoon lies horizontally on the inner side of the egg surface. Since this is not an artifact of the technique, as will be shown later, the spermatozoon has, then, undergone a rotation from the original perpendicular position in which it attached and fused to the horizontal one it has now assumed.

In the next image the spermatozoon can be seen to be continuing to rotate (Fig. 20). Previously it was positioned parallel to the egg surface, and now it is beginning to rotate further so that its posterior end enters the egg cytoplasm first. This rotation seems to be initiated at the anterior part of the spermatozoon. The cortex can clearly be seen to be intimately associated with the membraneless spermatozoon and separating it from the egg cytoplasm. Finally, the spermatozoon completes its rotation through the egg surface so that its centriolar end faces inwardly and the anterior part of the sperm nucleus remains attached to the cortex (Fig. 21). The spermatozoon then detaches into the cytoplasm, where fusion with the female pronucleus will occur.

In Fig. 21, just above the fully rotated spermatozoon, a blemish or scar in the previously uniform cortex can be observed. This is the result of the penetration of the spermatozoon through the cortex. After detachment, these scars persist (Fig. 22); they average 2.12. μm, sufficient to permit the passage of the spermhead. The fibers circumscribing the scar are composed of the same diameter as those of the cortical network. Interestingly, there are about 20 270 nm particles incorporated into these fibers; they are reminiscent of the particles seen in the earlier images and their reappearance is not understood.

One could imagine that the tight flattening of the egg surface to the polylysine-coated plate would push an underlying spermatozoon through the egg surface, even though the spermatozoon had not really penetrated the egg. As a control against this

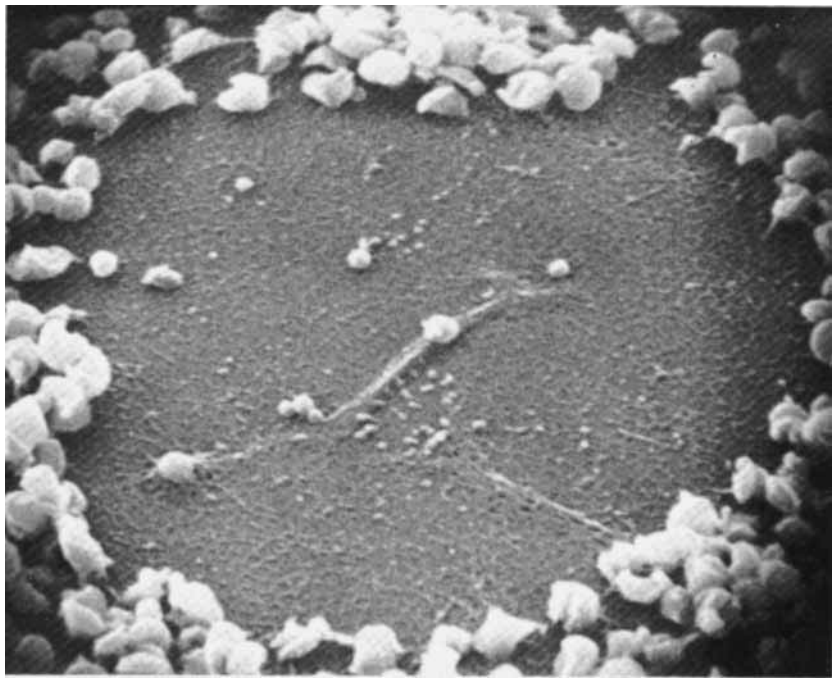

Fig. 16. The first observable effect of fertilization on the inner surface. In the region where the cortical granules have discharged, the clustering of the 235 nm particles is observed. These particles probably correspond to the bases of the elongate microvilli seen in Fig. 7. The cortex is demonstrated here to be a uniform meshwork of 50–200 nm fibers. The triangularly-radiating thickening in the cortex may be the result of the contraction of cortical proteins, and may be responsible for the surface deformations observed on the outer surface. 6,500 ×.

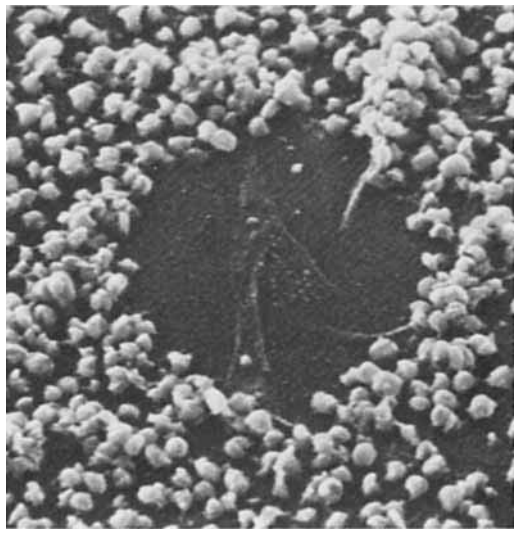

Fig. 17. The particles cluster as the microvilli interdigitate on the outer surface (see Figs. 8, 9). The triangular distortion in the cortex is thicker and covers a greater area; this is consistent with the increased magnitude and diameter of the surface deformation observed on the outside. 3,000 ×.

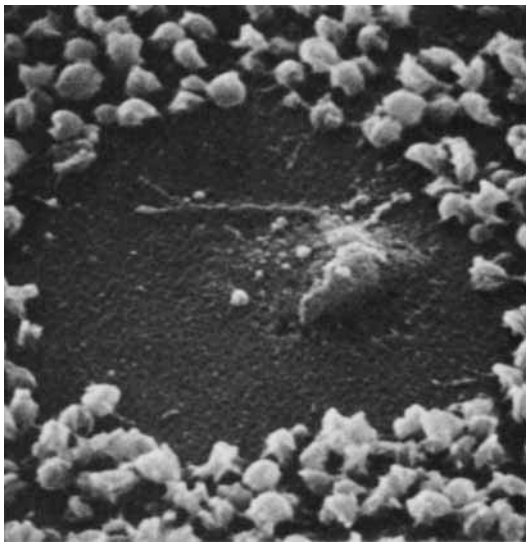

Fig. 18. The spermatozoon appears on the inner surface in the patch devoid of the cortical granules. Notice that it is lying on the egg surface. 5,000 ×.

possible artifact, the following experiment was performed. Sperm were glued onto a polylysine-coated plate, and then either fertilized or unfertilized surfaces were glued on top. If the flattening were forcing the egg surface around the spermatozoon, it should be possible to observe the sperm through the egg surface. In no instance could the sperm or an outline of a sperm be discerned; consequently, we argue that these results are the actual sperm movements through the egg surface and not an artifact.

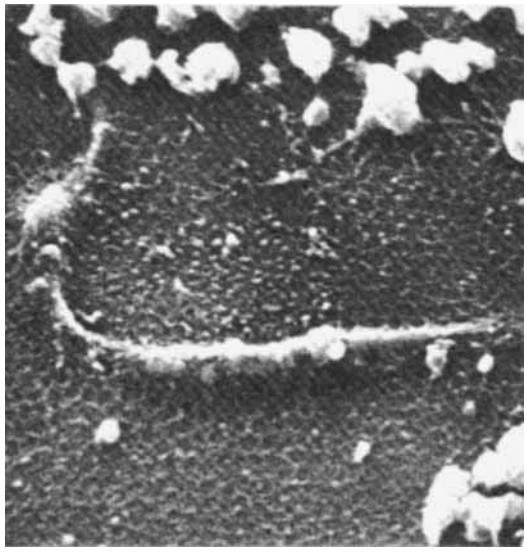

Fig. 19. It is apparent here that the spermatozoon is lying between the plasma membrane and the cortex. It is separated from the egg cytoplasm by the cortical fibers. 6,000 ×.

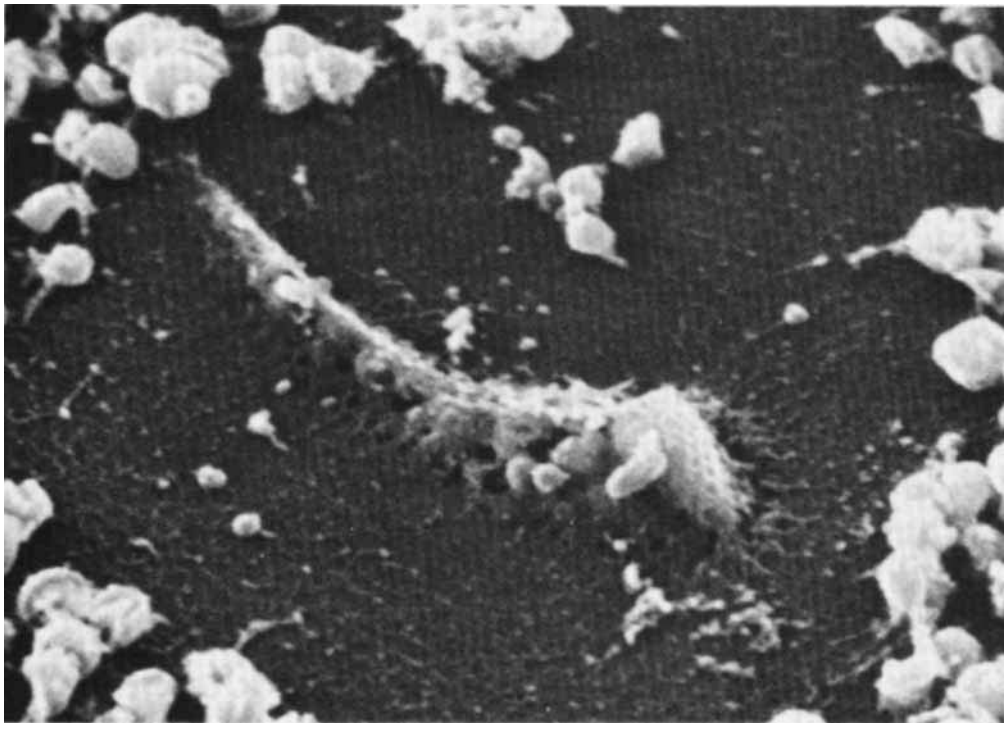

Fig. 20. The intimate association between these cortical elements and the membraneless spermatozoon can be observed as the spermatozoon continues to rotate through the egg surface. This rotation appears to start at the anterior end of the sperm. 7,500 ×.

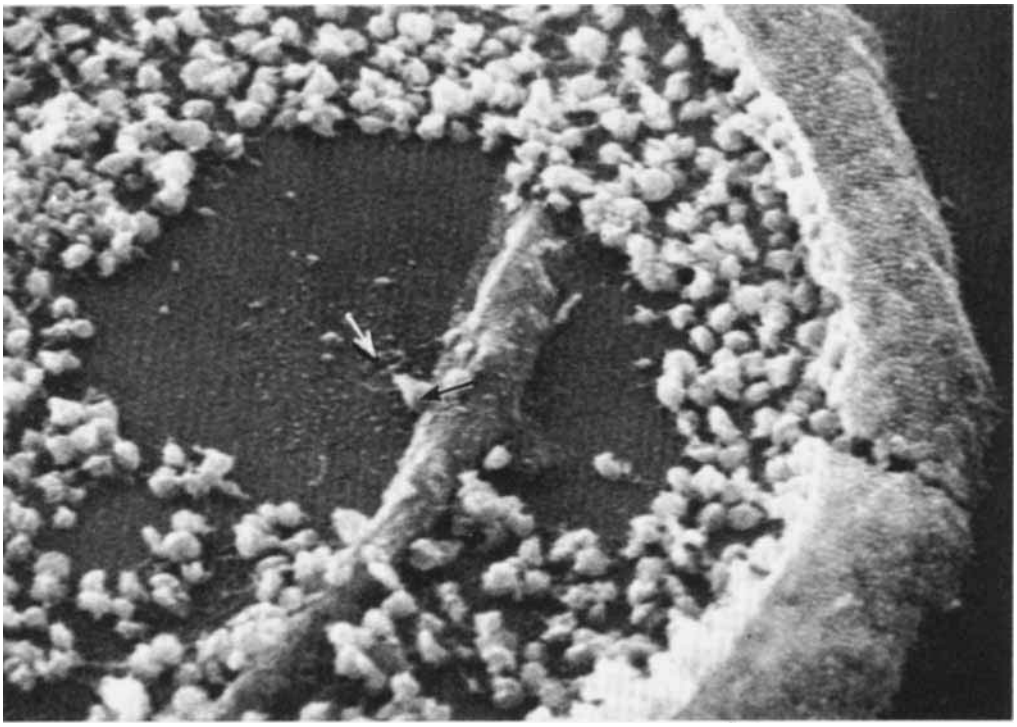

Fig. 21. The spermatozoon (black arrow) has completed its rotation and is about to detach into the egg cytoplasm. The scar (white arrow) on the cortex through which it penetrated can be observed just above this spermatozoon. 3,000 ×.

FERTILIZATION AT THE EGG SURFACE: A SYNTHESIS

The external and inner images of the spermatozoon can be used to reconstruct the surface events associated with attachment, fusion, rotation, entry, and detachment from the cortex. These results corroborate and add new information to the documentation of Wilson and Leaming with the light microscope (10), and of Longo and Anderson with the transmission electron microscope (TEM) (11).

The first activity of fertilization is the discharge of the acrosome of the spermatozoon, as can be seen from the diagram and the figures (Fig. 2, Diag. A). It has been shown that when the acrosomal vesicle fuses with the plasma membrane of the spermatozoon, a vesicle is formed and sloughed off (8). The base of the sperm at this stage has such a vesicle. The extended acrosomal filament, probably by an actin contraction (12), brings the spermhead into intimate contact with the egg surface. The spermatozoon attaches perpendicularly, and shows no specific preference for either the tips or the bases of the vitelline papillae. Fifty nm strands, first observed by Tegner and Epel (13), may be involved in the attachment of the sperm to the egg surface; these strands probably originate from the vitelline sheet. The continuous vitelline sheet is found to be either solubilized or torn adjacent to the tip of the acrosome, perhaps to permit close association of the membranes in preparation for cell fusion.

The stages of fusion of the membranes of the egg and the spermatozoon may be traced, taking the microvilli as markers of the surface of the egg; the surface of the

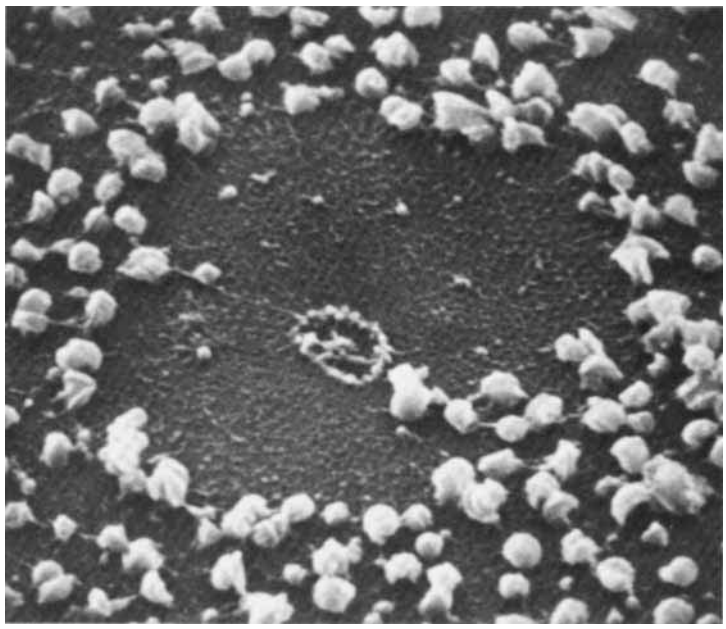

Fig. 22. The scar in the cortex, through which the spermatozoon penetrated, persists after the sperm has detached into the egg cytoplasm. Small particles are incorporated into the circumscribing fibers. 5,000 ×.

spermatozoon is smooth. At the first moments of fusion, the surface of the egg is uplifted in a small cone (Figs. 4, 5; Diag. B). When the membranes fuse, the internal mass of the spermhead is seen enclosed in a column which is proximally egg membrane and distally sperm membrane (Fig. 6, Diags. C, D). As the fusion progresses, the overall impression is that of an increase in area of the membrane around the spermhead, which is observed as wrinkling and slackness of the membrane (Fig. 7, Diag. E). Next, the microvilli cluster and elongate around the spermhead as it is sinking into the egg (Figs. 7, 8, 9; Diag. F); the membrane over the most distal part of the spermhead, around the midpiece, appears slack and convoluted. These elongate microvilli interdigitate over the penetrating spermatozoon as the slackness of the sperm-derived membrane diminishes (Fig. 9; Diag. F). The spermhead and midpiece disappear from the outer surface, while the surrounding region on the outer surface is greatly deformed (Figs. 11, 12; Diag. G). The vitelline appears to separate from the plasma membrane at this moment, as evidenced by the depression in the tips of the vitelline papillae. Finally, the tail and its membrane, which is now taut, are seen protruding from the egg surface as the vitelline sheet elevates into the fertilization coat (Fig. 13; Diags. H, I).

Cortical granule discharge is initiated at the time when membrane fusion between the gametes occurs. On the exterior, the spermatozoon is surrounded by a cluster of about 20 elongate microvilli with a basal diameter of 2.5 μm. The cortex reveals 20–25 particles arranged in a circle 2.6 μm in diameter. Since the size, diameter, frequency, and time of appearance correspond, it is postulated that the particles are associated with the bases of the microvilli. Longo and Anderson (11) described rod-containing vesicles of the same dimensions associated with the basal part of the fertilization cone; the 235 nm particles may correspond to the vesicles seen in the TEM (Figs. 7, 15; Diag. E). Next, the elongate

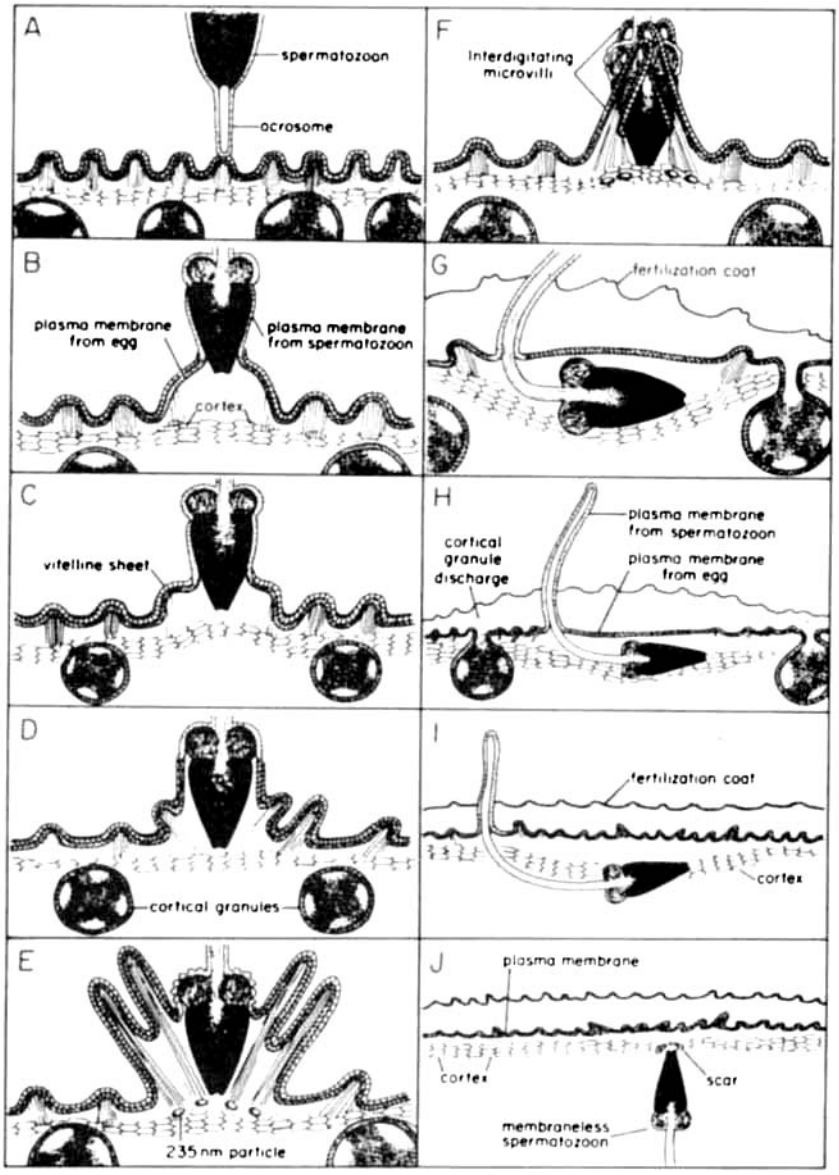

Diagram I. The rotation of the spermatozoon through the egg surface. A) The spermatozoon initially contacts the egg with its elongated acrosomal process. These processes appear to shorten and thicken as the spermhead approaches the egg surface. B) Prior to membrane fusion, the vitelline sheet nearest to the anterior tip of the spermhead separates slightly. Then, as fusion occurs, the egg surface bulges up at the site of contact. C) The egg surface continues to bulge up at the site of fusion, as a surface deformation spreads in diameter and magnitude. D) The membrane derived from the egg, apparent because of the presence of microvilli, rises up around the spermhead. This composite membrane loosely covers the sperm nucleus. E) The microvilli adjacent to the sperm elongate around the sperm. The egg membrane has continued to rise up around the spermatozoon, resulting in a loose and convoluted appearance of the smooth sperm-derived membrane. There appears to be 235 nm particles associated with the bases of the microvilli. F) The elongate microvilli interdigitate and enmesh over the sperm as it is forced onto the underlying cortex. The slackness of the membrane is beginning to

be resorbed. G) As the cortical granules discharge, and the vitelline sheet separates from the plasma membrane, the spermatozoon rotates to lie between the membrane and the cortex. It is not free to directly penetrate the egg cytoplasm due to the fibrous cortex. H) The membrane around the spermatozoon resumes its taut appearance as the membraneless spermatozoon establishes intimate associations along its length with the cortical fibers. I) The spermatozoon rotates through the cortex, beginning at its anterior end. J) Finally, the spermatozoon is detached from the cortex into the egg cytoplasm after having circumscribed a 180° rotation through the surface layers. The scar in the cortex persists after the spermatozoon had detached.

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microvilli interdigitate over the spermhead as these particles aggregate in the center of the patch; this is another correlation between the particles and the microvilli (Figs. 9, 16; Diag. F). The perpendicularly attached spermatozoon has not yet begun to penetrate the surface, and it is significant that higher magnification images reveal a circular structure reminiscent of the tip of the sperm nucleus in the center of the cortical distortions and particles. This is consistent with an association between the anterior tip of the spermatozoon and the cortex prior to the onset of rotation and penetration.

The fusion of the membranes of the gametes also seems to initiate a surface deformation which spreads in magnitude and diameter as the spermatozoon penetrates. This surface contraction can be observed from the outside as the spreading surface deformation surrounding the site of penetration (Fig. 11), and by the clustering of the elongate microvilli and their subsequent interdigitation. The indentations in the tips of the papillae of the vitelline sheet observed in Fig. 11 probably occurred when the underlying microvilli withdrew their support from this sheet – another indication of an active surface. The cortex at these stages also reveals distortions analogous to those observed on the outer surface. When the microvilli cluster around the spermatozoon (Fig. 7), the cortical fibers in the center of the patch show a triangularly-radiating thickening (Fig. 16). Then, as the microvilli interdigitate and the surface deformation spreads (Fig. 9), this cortical thickening increases in fiber size and area (Fig. 17); this is consistent with a contraction of the cortex causing the surface deformation. It is interesting to speculate about the triangular nature of these cortical thickenings. The cortical fibers circumscribe roughly hexagonal polygons which cover the entire inner surface of the egg. When the egg is required to rapidly deform its surface, radiating from the attachment site of the sperm, a contraction and relaxation of adjacent polygons might be employed. This fashion would rapidly and economically distort the surface into the observed spreading waves (Fig. 11). This model, based on a hexagonal array, would result in the observed triangular thickenings of the cortex.

As the deformation spreads, the vitelline sheet appears to drape loosely upon the egg surface as if the contour of the vitelline sheet and the plasma membrane no longer corresponded; it is at this stage that the vitelline sheet is probably detached from the plasma membrane (Diag. H). This rapidly spreading deformation, probably caused by a contraction of the cortex, may be required for a uniform detachment of the vitelline sheet from the egg plasma membrane, may be involved in a rapid block to polyspermy, and may participate in releasing the cell from the metabolic constraints of the unfertilized egg.

It is interesting to further speculate about the elongation of the microvilli observed at fertilization, and also during metabolic turn-on. This may be another example of the mechanism proposed by Tilney et al. (12) for the elongation of the acrosomal filament: conversion of a profilamentous form of actin into microfilaments of F-actin.

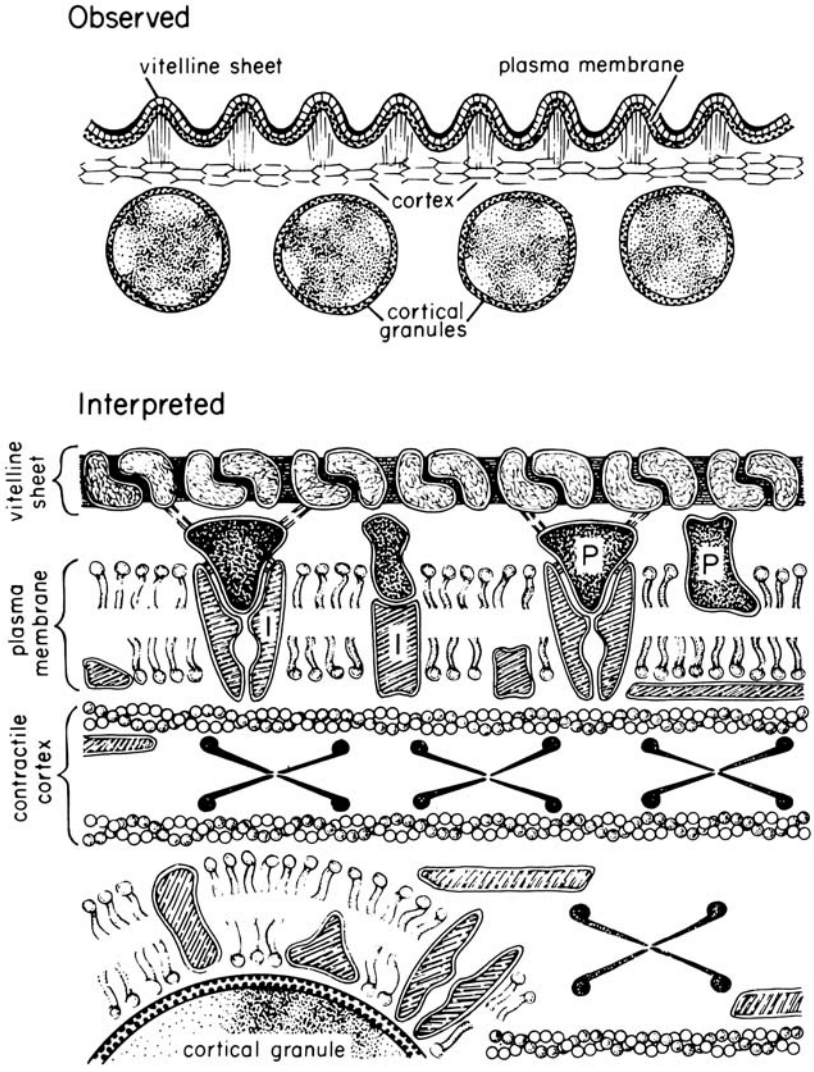

Diagram II. The surface layers of the unfertilized sea urchin egg. A) Observed. B) Interpreted (see text for description).

P = Peripheral membrane protein; the metabolic derepressor.

I = Integral membrane protein(s): drawn schematically as a channel permitting new syntheses and activities when the stopper, the metabolic derepressor, is pulled.

The spermatozoon rotates while penetrating the egg surface to, at first, lie upon the inner surface of the egg (compare Figs. 7, 11; Diags. F, G). This movement may be accomplished by either or both of 2 methods. The first method would be based on a surface tension concept. It argues that after membrane fusion the sperm and egg are bounded by a common plasma membrane. If the elements holding the spermatozoon perpendicular during fusion are removed, the tendency to minimize surface area might be the motive force in causing the sperm to lie between the plasma membrane and the cortex. The sperm

is not able to directly penetrate the egg cytoplasm due to the barrier of the cortex (compare Diags. F, G). Alternatively, although not exclusively, the components responsible for the interdigitations of the microvilli, probably the cortical fibers, might force the sperm into the egg cortex by an enmeshing and squeezing action.

A further correlation between the inner and the outer events on fertilization is available at this step. As the sperm disappears from the outer surface, a bump of the same size and shape appears on the inner surface (Fig. 18). Since the cortex has been measured to be maximally 0.5 μm thick, the sperm would not be expected to be able to "hide" in the surface. The entire spermatozoon, less its plasma membrane, enters the egg and appears to be intimately attached to the cortex (Figs. 19, 20; Diag. I).

The spermatozoon continues its rotation as it journeys into the cytoplasm. After the initial contact with the cortex, which seems to occur when the microvilli interdigitate with the sperm in the perpendicular orientation, the sperm lies upon this cortex and establishes intimate contact with the fibers along the length of the head, midpiece, and axoneme (Figs. 19, 20; Diag. I). It then continues, by penetrating through the cortex, to be oriented 180° from its original attachment (Fig. 21; Diag. J). This movement is initiated at the anterior end of the spermhead, and may be caused by contractile elements in the cortex. Finally, the sperm detaches from the cortex into the cytoplasm, leaving a scar in the cortex through which it had penetrated (Fig. 22, Diag. J). The small particles found in the earlier cortical images reappear incorporated into this scar. Since the elevated fertilization coat obscures the egg surface, it is not clear if they have a complement of microvilli on the outer surface.

It is possible to make an approximate estimate of the time required for the sperm to penetrate through this surface, if certain assumptions are made. The method for determining the length of time required from the moment of membrane fusion until detachment of the spermatozoon depends upon the size of the area without cortical granules. It has been shown that the cortical granules discharge at a uniform rate of about 10 μm/sec (14). However, it appears that the smallest region devoid of the cortical granules at the earliest stage is about 10 μm. It may be that the sperm initiates the discharge of a region which then spreads at the observed rate. Now by determining the diameter of the patch at the earliest stages and at the latest stage, and using the figure of 10 μm/sec, it is possible to estimate the time required. This calculation assumes that the isolation medium stops both the discharge of the cortical granules and the movement of the spermatozoon at the same time. The largest areas are 23 μm while the smallest are 10 μm. The difference in diameter of 13 μm results in a 6.5 μm radius. At the given rate of discharge, this would imply that 0.65 sec are required from the time of fusion for penetration, rotation, and detachment. This predicts, then, that the movement is very rapid indeed. In the work of Longo and Anderson with the TEM (12), sperm were observed on the outside or just inside of the egg, but never in the process of penetrating, again arguing for a very rapid movement. In this work vast numbers of egg surfaces were examined to observe the intermediate stages of penetration, another indication that this movement may require less than 1 sec to be accomplished. It is interesting to note that in the cinematographic analysis of fertilization by N. Epel and D. Epel (personal communication) the attached spermatozoon writhes about for 15–20 sec, then stands perpendicular to the egg surface as the fertilization coat elevates. It is possible that membrane fusion does not occur until the spermatozoon stands erect, which then may initiate the cortical granule discharge as the spermatozoon is engulfed. Their data argues for a time span of about 20

sec until the spermatozoon enters the egg. However, they start their timing at the movement of attachment, whereas here membrane fusion is the beginning point of time measurement. Additionally, Dan (14), studying the more transparent Japanese sea urchin eggs with the phase contrast microscope, reports that it takes from 2–4 min for the 40 μm sperm tail to enter the egg.

It is possible to reconcile these data into a tentitive time course for the entry of the spermatozoon. The sperm attaches to the egg surface, and after some time, about 15–20 sec, membrane fusion occurs. Then the spermatozoon is rotated through the surface concommitant with the elevation of the fertilization coat and the rise of the fertilization cone; the rotation is reported here to take a very short time, perhaps less than 1 sec, whereas the wave of cortical granules' discharge requires about 20 sec to spread over the egg surface. Then the entire length of the sperm tail is pulled into the egg, after a total elapsed time of from 2 to 4 min.

THE EGG CORTEX

The existence of a thick and rigid layer underlying the plasma membrane, called the cortex, has been postulated since the turn of the century (15). Chambers in 1917 (16) inferred the existence of such a gelated layer, 1–2 μm thick, on the basis of micromanipulation studies. Various ingenious biophysical studies, reviewed by Hiramoto (17), have deduced properties of this layer, and Mitchison (18) determined it to be about 2 μm thick in the living egg. Transmission electron microscopy has failed to describe such a structure (19, 20). Vacquier (5) has described interconnections between the cortical granules in the undersurface of unfertilized egg in the SEM if they were first fixed and subsequently isolated. The existence of a layer of secretory vesicles, the cortical granules, implies some structure holding them to the plasma membrane. Additionally, they resist displacement after centrifugation, although the rest of the cytoplasm is stratified.

Here it is possible to examine the cortex directly, as in Fig. 21. These cross-sectional images reveal a cortex of about 0.5 μm thick, with only the cortical granules well-resolved. This measurement is lower than the other estimates, which can be explained in 2 ways. Measurements on living eggs in the light microscope are difficult and probably somewhat inaccurate at this extreme. Furthermore, the cortex might exclude cytoplasmic organelles on which the estimate was made. Alternatively, the dehydration and drying steps employed for the SEM might shrink the structure by the stripping of water; this could also account for the discrepancy. Additional information about a layer underlying the plasma membrane is given in images such as that in Fig. 20, where an irruption of the sperm nucleus and axoneme through the undersurface of the membrane makes clearer the presence of a fibrous layer over that surface. Furthermore, the regularly arrayed polygons (Fig. 16), which are capable of distortions, permit speculation about movement in this structure.

It was important to determine if the fertilized egg also has a cortex of similar morphology to that observed at fertilization. To visualize this the fertilization coat was removed, and the tops of affixed eggs were sheared off. The low magnification image (Fig. 23) demonstrates that the entire inner surface of the egg is enveloped by this structure. The following higher magnification reveals 50–200 nm fibers coalescing and ramifying to form varying size bundles (Fig. 24). The thickness ranges between 0.2–0.5 μm, with overlapping of the fibers accounting for some of the variation. These eggs were fixed 10 min after fertilization; however, the cortex may be found at any stage of development.

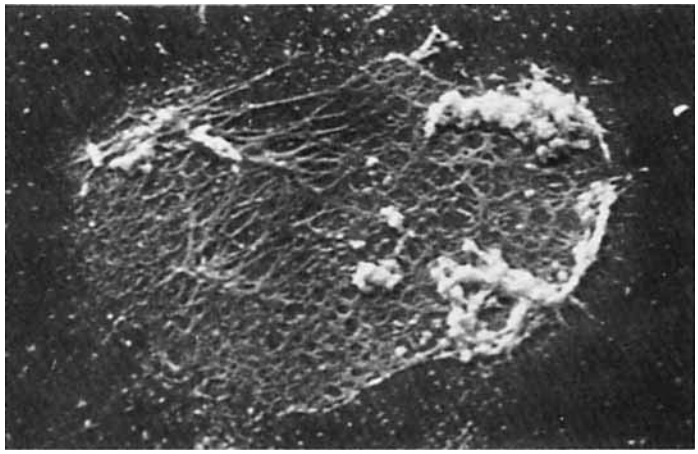

Fig. 23. After completion of the cortical granule discharge, the cortex appears as an overlapping network of fibers 0.2–0.5 μm thick. This specimen was isolated 10 min after fertilization. 1,000 ×.

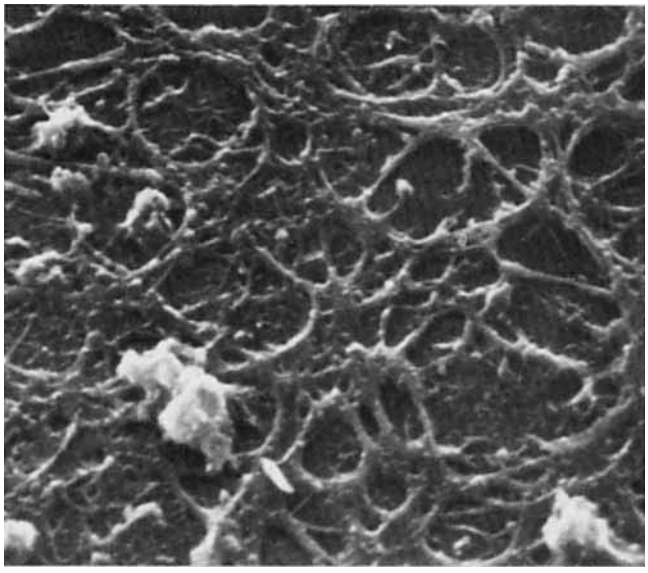

Fig. 24. This higher magnification image demonstrates that the cortical fibers still measure about 50–200 nm. These fibers coalesce and ramify, giving the impression that the fibers are composed of multiple units. The smallest of these units appears to be about 10 nm. 25,000 ×.

The many examples of movement at the moment of fertilization shown in this work makes attractive the idea of a contractile surface layer. Mabuchi (21) has shown the presence of a myosin-like ATPase in isolated surfaces from sea urchin eggs. Recent work by A. Spudich and J. Spudich (personal communication) has demonstrated the presence of actin in sea urchin egg surfaces. Furthermore, the 50–200 nm fibers are extracted by 0.6 M KI, leaving the rest of the surface intact. This KI extract has been shown by gel electrophoresis to contain actin (courtesy of A. Spudich). Actin has been shown to be

attached to the inner face of the membrane in other cells as well (22). This contractile layer may be responsible for surface changes involved in the block to polyspermy, in the cortical granule discharge, in facilitating the entry of the spermatozoon, and in metabolic activation.

IMPLICATIONS FOR MEMBRANE FUSION

As a system for examining the relative diffusibilities of fusing membranes, fertilization is unexcelled. In this system there are natural surface markers for both the sperm and the egg. The membrane from the spermatozoon is smooth and devoid of surface projections, whereas the egg membrane is completely covered with microvilli (Fig. 3). It is therefore possible to determine the cellular origin of any given region of the membrane at fertilization.

At the earliest moments of membrane fusion, the egg bulges up around the tip of the spermatozoon (Fig. 5; Diag. B). Subsequently, the egg membrane, i.e., the membrane possessing microvilli, rises up around the sperm nucleus, resulting in a loose appearance in the distal sperm-derived membrane (Fig. 6; Diag. D). Then as the sperm penetrates into the egg membrane, the slack sperm-derived membrane is taken up. The usually taut appearance is resumed and, finally, as the sperm's membrane mixes with the egg's, the smooth patch of membrane disappears (compare Diags. D–J).

When the gametes initially fuse, an excess of membrane, here characterized by the loose smooth membrane covering the spermatozoon, may have resulted from the diminished exposed surface area. This slackness may be exaggerated by the flow of egg-derived membrane up around the sperm nucleus. Then, as the sperm penetrates into the egg (a situation which should result in an even greater excess of membrane if this were due to the diminishing surface area), the membrane slackness is resorbed and it begins to assume the normally taut appearance. Consequently, it is possible to argue about the relative diffusibility of the 2 membranes rather than about the size of the exposed area to be covered. It appears that the egg membrane is more mobile than that of the sperm. The egg membrane can flow around the sperm nucleus and displace the sperm membrane, and it is only after some time that the smooth sperm-derived membrane diffuses into that of the egg. Eventually, after intermixing, no region of smooth membrane is found.

In addition to the difference in relative mobility of the membranes, these events may involve absolute decreases or increases in the area of the fusing membranes. It appears that the egg membrane expands at the site of fusion, while the membrane surrounding the spermatozoon appears to decrease in area. The apparent stretching and contraction of membrane area, observed whenever a cell changes shape, is most common. The current fluid-mosaic model (23) of membrane structure does not appear to allow for the extensive and rapid changes in area seen in many kinds of cell behavior. Probably, these phenomena will find their explanation in continuing studies of exchange of membrane components between the interface and interior of the cell.

THE OUTER SURFACE

The outermost surface of the unfertilized egg, excluding jelly, is the vitelline sheet, which has been shown by Tegner and Epel (13) to be the foundation of the fertilization coat. Connecting this sheet to the plasma membrane is a peripheral membrane component which is responsible for maintaining the unfertilized egg in a metabolically inactive state

(4). Perhaps the conceptually simplest demonstration of this crucial role of the outer surface is an experiment where the outer surface is physically torn off of the egg. Protamine-coated glass fibers will adhere to the outer surface of the egg, and upon shaking will tear the vitelline sheet from the plasma membrane. Eggs so treated have been shown to be turned-on by cytological, electrophysiological, and ultrastructural criteria. Additionally, chemical agents which will not penetrate, or only slowly penetrate, through the plasma membrane, such as isoosmotic glycerol or urea, will solubilize these outer layers, resulting in the turn-on of the egg. Finally, ammoniated sea water at basic pH, which has been shown by Mazia and his coworkers to cause turn-on, also removes these outer components, releasing the egg from its metabolic constraints.

It has been shown that the vitelline sheet is sensitive to sulfhydryl reducing agents such as dithiotheitol (24). An egg so treated is found not to be turned-on, but has a sprinkling of particles which are removed by any agents which will turn the egg on. Consequently, it has been possible to conclude that it is not the vitelline sheet per se, but rather the component attaching the vitelline to the membrane, that is responsible for the metabolic derepression observed at fertilization and during turn-on. Finally, the reciprocal experiment of removing this attachment site (and turning the egg on) without solubilizing the vitelline sheet is possible by treating the eggs with mM concentrations of ethylamine at basic pH (4). This is analogous to the events at normal fertilization. A convincing experiment that this outer peripheral membrane protein controls the metabolic level of the egg is shown in the work of Johnson and Epel (25). They succeeded in adding the ammonia-solubilized protein back to turned-on eggs, with the result of shutting the activated protein synthesis off. It is imagined that these peripheral components are separated from the plasma membrane when the vitelline sheet separates from the surface. This detachment may be physically mediated by a contraction of the cortex, or it may be accomplished by a release of proteolytic enzymes from the cortical granules.

RECAPITULATION AND SUMMARY

The accumulated evidence from the morphological examination of the penetration of the spermatozoon through the egg surface, the chemical and physical treatments that induce turn-on, and the biochemical analyses of isolated surfaces is compiled here to review the alleged functions of each layer (see Table I for review). Beginning from the outer surface (see Diag. II), the vitelline layer serves as the foundation for the fertilization coat. It also seems to hold the microvilli of the unfertilized egg in the characteristic array. It is solubilized by nonelectrolyte treatments, ammonia, and disulfide reduction, and can physically be torn from the surface.

Connecting the vitelline layer to the membrane are peripherally associated membrane proteins which serve as the metabolic derepressor at fertilization. In the unfertilized state these components maintain the cytoplasm in an inactive state. Their removal activates DNA synthesis, protein synthesis, polyadenylyation of mRNA, K+ conductance, chromosome cycles of condensation and decondensation, and other new metabolic activities normally stimulated at fertilization. Unpublished information also has demonstrated that these particles are involved in the slow, permanent block to polyspermy (26).

The plasma membrane in this cell serves as an ionic barrier capable of permeability changes relative to the metabolic state of the egg. The vitelline sheet is attached to it in the unfertilized egg, and the cortex must also be included in a complete model of the surface complex. This membrane is also able to undergo the transition from 1) being capable

of fusing with sperm, to 2) a nonfusible state, to prevent the entry of supernumerary sperm.

The cortex, previously unconfirmed by electron microscopy, is shown in this study to exist as a fibrous matrix beneath the plasma membrane and probably attached to it. The existence of this structure was postulated from biophysical and micromanipulation studies of the rigidity of the surface, even though early electron microscopy failed to find it. This cortex, which contains myosin and actin, seems to cause a spreading surface deformation at fertilization which may be involved in the secreation of the cortical granules, in the detachment of the vitelline sheet from the egg surface, and perhaps in the rapid block to polyspermy. Attached to this cortex in the unfertilized egg are the secretory cortical granules. These granules fuse membranes with the plasma membrane at fertilization discharging their contents into the perivitelline space. The structural contents laminate the undersurface of the vitelline sheet, elevating and hardening it to form the fertilization coat. Additionally, proteolytic enzymes that are released detach the unsuccessful sperm attached to the fertilization coat, and may aid in the release of the metabolic derepressor from the plasma membrane. Furthermore, the hyaline layer required for proper development is deposited on the egg surface at this stage.

The image of the cell surface of the unfertilized egg does not differ so much from a model that could be assembled from various observations on other cells. The advantage of the egg is that the complex structure is explicit and well-displayed. The remaining generalized questions regarding surface biology include questions about: the mode of action whereby the metabolic derepressor activates new syntheses; the behavior of membrane components permitting permeability, fluidity, and fusibility alterations; and the attachment of the cortex to the surface complex and the regulation of its behavior.

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